Methylation is Involved in *Arabidopsis thaliana* Recovery from Geminivirus and Prevention of Geminivirus Seed Transmission

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Introduction

While most plant viruses are RNA viruses that replicate in the cytoplasm of the host cells, members of the *Geminiviridae* family are unique in that they are DNA plant viruses. Geminiviruses possess either monopartite or bipartite circular single-stranded DNA (ssDNA) genomes of 2.5-3 kb, that replicate in the nucleus through double-stranded DNA (dsDNA) intermediates (Bisaro, 1996). The genome components of bipartite geminiviruses are almost entirely different in sequence except for a 200-250 bp conserved region. The geminiviruses used in my study are the bipartite *Cabbage leaf curl virus* (CaLCuV) of the genus Begomovirus, and the monopartite *Beet curly top virus* (BCTV), of the genus Curtovirus (Bisaro, 1996). Geminiviruses are typically transmitted from plant to plant via insect vectors such as the whitefly (Begomovirus) or leafhopper (Curtovirus) (Bisaro, 1996). They infect several plant species and for my studies I focused on the model plant host *Arabidopsis thaliana*. Geminiviruses replicate predominantly through rolling circle replication and rely on the host replication machinery to accomplish amplification and transcription (Bisaro, 1996). The AL2 protein (Begomoviruses) is a transcription factor that is responsible for the expression of late viral genes (Sunter and Bisaro, 1997). For Curtoviruses, the related L2 protein is not a transcription factor, but acts as a suppressor of plant silencing pathways, as does AL2. One mechanism of silencing involves AL2 and L2 interacting with adenosine kinase (ADK), which is responsible for the phosphorylation of adenosine to AMP (Fig.1). ADK activity is also required for efficient operation of the methyl cycle (Wang et. al., 2003).

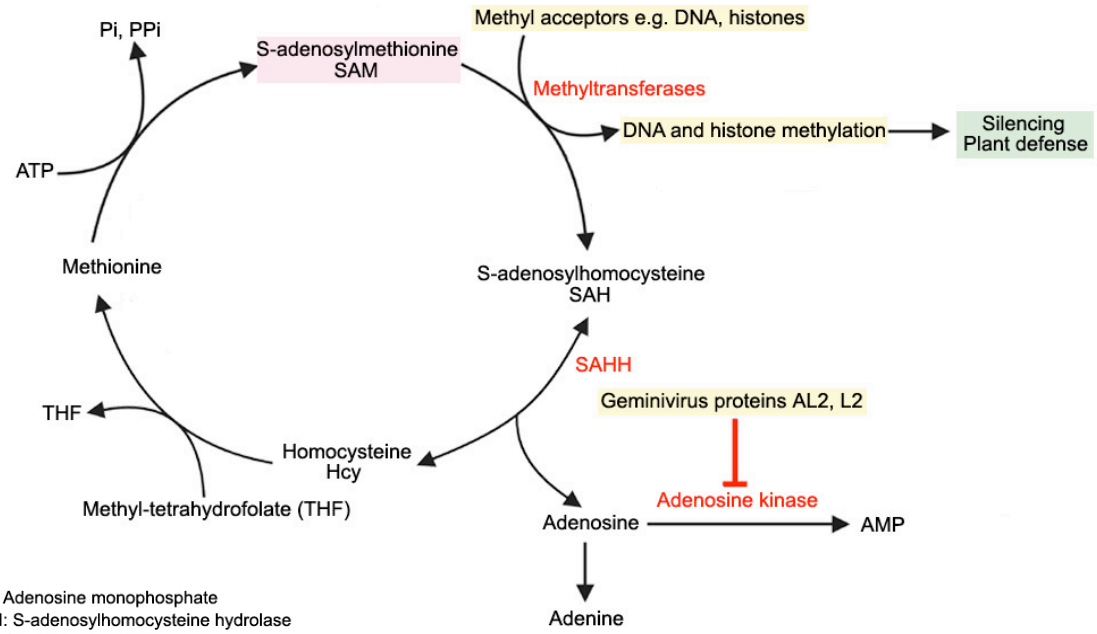

Fig. 1: ***Arabidopsis* methyl cycle.** The methyl cycle generates SAM, which is the methyl donor for most transmethylation reactions. AL2 and L2 inhibit the methyl cycle by blocking the conversion of adenosine to AMP, which drives the equilibrium towards Hcy. The equilibrium typically lies in the opposite direction because SAHH is a poor enzyme.

Plants use two main RNA silencing pathways for defense against geminiviruses. Post transcriptional gene silencing (PTGS) involves small interfering RNAs (siRNAs) to repress gene expression by directing the cleavage of homologous mRNAs in the cytoplasm (Vaucheret, 2008) (Fig. 2). Plants deficient in PTGS pathways are usually more susceptible to infection by plant viruses. PTGS is induced by dsRNA, and two classes of proteins, Argonautes (AGO) and Dicer-like proteins (DCL), are key players in the pathway. In PTGS in Arabidopsis, DCL2 or DCL4 process dsRNA into 21-22 nucleotide (nt) siRNA duplexes, and one strand of the siRNA in incorporated into an RNA induced silencing complex (RISC) which contains an AGO1 protein which is also

an endonuclease (slicer). The siRNA programs RISC to cleave mRNAs with complementary sequence (Vaucheret, 2008) (Fig. 2).

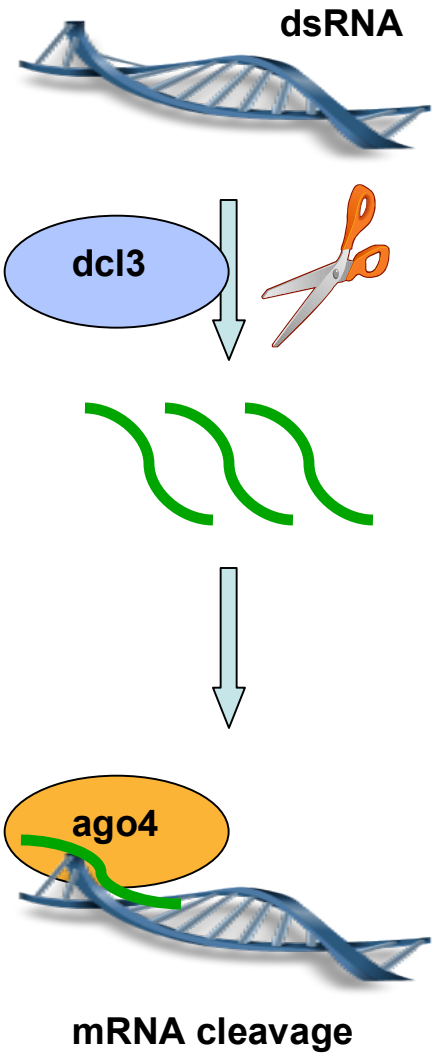

Fig. 2: PTGS pathway. Dicer-like protein (DCL2 or DCL4) cleaves exogenous or endogenous dsRNA into siRNAs which are loaded into a RISC complex that contains an AGO1 endonuclease (slicer). One strand of the siRNA directs cleavage to homologous mRNA sequences.

The second pathway is transcriptional gene-silencing (TGS), which results in the methylation of target DNA sequences in the nucleus. This pathway uses similar RNA silencing machinery, except in this case dsRNA is processed by DCL3 into slighter larger siRNA (~24 nt) which enter a RISC complex that contains AGO4. The function of this RISC complex is to recruit methyltransferase proteins to specific DNA sequences (Qi et. al., 2006). Methylation of DNA and associated histone proteins is strongly correlated

with reduced transcription. One of the mutants used in this study is deficient in *ago4*, which is used in *Arabidopsis* siRNA production to suppress viral transcription.

Methylation is directed by siRNAs in cooperation with the ADK methyl cycle to methylate histones and viral DNA with a variety of methyltransferases (Wang et. al., 2005) (Fig. 3).

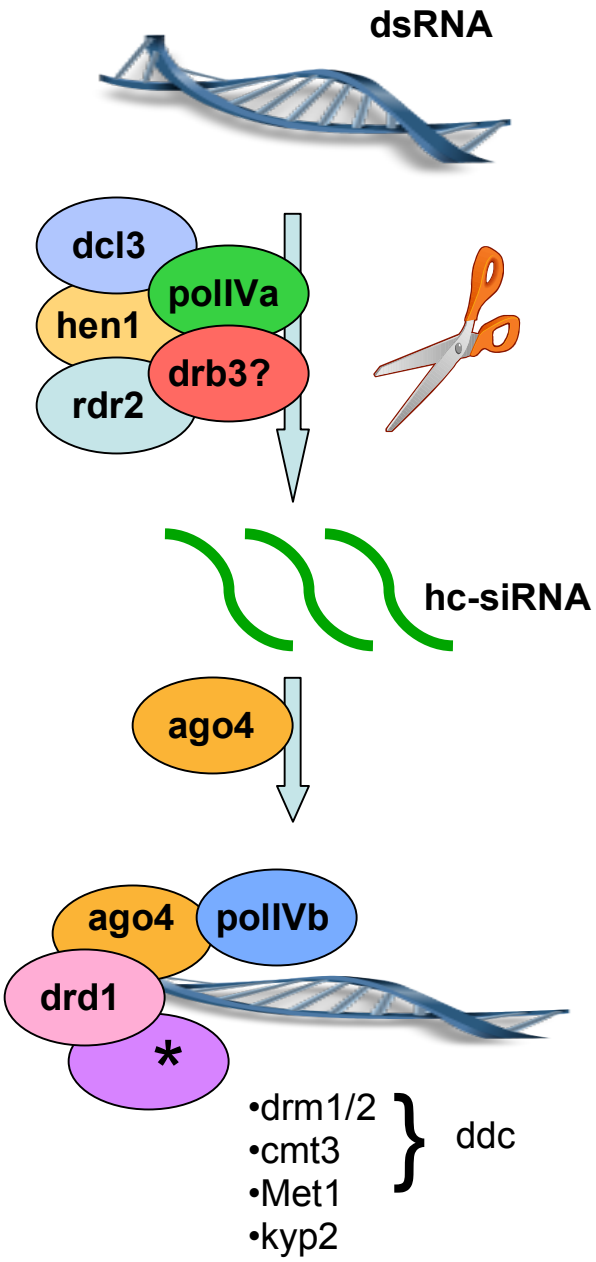

Fig. 3. The TGS pathway. Exogenous or endogenous dsRNA is processed into heterochromatin siRNAs (hc-siRNA) by a complex that contains DCL3. These siRNAs are then loaded by AGO4 into another protein complex which carries out methylation of viral DNA and histones for silencing. Mutants used in this study include *polIVa/b*, *drb3*, *drm1/2*, *cmt3*, *kyp2*, and *ddc*. Preliminary data suggests that *drb3* is also involved in methylation.

The process of viral DNA and histone methylation is carried out by many proteins, but this study will only be testing a handful of them. AGO4 has been described above. PolIV is involved in siRNA directed *de novo* cytosine methylation in *Arabidopsis*. Loss of PolIV brings about the absence of most cytosine methylation thus suggesting that it is

integral in producing the siRNAs used to carry out genome methylation (Pikaard, et. al., 2005). DRB3 is a dsRNA binding protein, which interacts with DCL proteins and likely facilitates the loading of siRNAs into RISC complexes (Hiraguri, 2005). DRM1/2 and CMT3 are both cytosine methyl transferases and may be partially redundant. Their complete removal with a *ddc* mutant removes all *de novo* DNA methylation at non-CG sites while CG sites are controlled by MET1 (methyltransferase1) which is not used in this study. KYP2 (kryptonite2) is a histone 3 lysine 9 (H3K9) methyltransferase and is also involved in heterochromatin methylation.

It is well-established that the PTGS pathway acts to degrade RNA virus transcripts and genomes, as well as the transcripts of geminiviruses. However, the Bisaro laboratory has recently shown that methylation of the geminivirus genomes, leading to transcriptional gene silencing, is an important defense against these DNA viruses (Bisaro et al., 2008). Methylation-deficient mutant plants show enhanced susceptibility to geminivirus infection and, and viral genomes obtained from these mutants show reduced methylation (Bisaro, 2008). Furthermore, that geminivirus AL2 and L2 proteins can block the the methyl cycle suggests that heterochromatin methylation is integral in the suppression of the viral genome.

We wished to ask further questions about methylation defense in *Arabidopsis*. One hypothesis tested is that the methylation pathway is required for plants to recover from geminivirus infection. Previous work has shown that *Nicotiana benthamiana* plants can recover from infection with BCTV L2 mutant viruses. Recovered tissue is nearly symptom-free and contains low levels of virus (Hormuzdi and Bisaro, 1995). Moreover, L2 mutant-infected plants show enhanced ADK activity, suggesting the involvement of

L2 in the suppression of ADK and implicating the methylation pathway in recovery (Wang et. al., 2003). Thus one goal of this study was to observe the behavior of two BCTV L2 mutant viruses on various methylation mutant *Arabidopsis* plants. It was predicted that wild-type plants would recover because of their ability to properly suppress the virus. However, methylation deficient plants (*ago4, cmt3, kyp2,* and *ddc)* were predicted to be unable to suppress the virus and would therefore still exhibit symptomatic tissue with the new plant growth (i.e. no recovery). We also predicted that virus extracted from recovered tissue would be hypermethylated. Conversely, viral DNA from tissue showing symptoms was predicted to have considerably lower methylation.

A second hypothesis is that plant methylation pathways are needed to prevent geminiviruses from invading the meristem. Most viruses are excluded from the meristem, and it has been shown that in the case of certain RNA viruses, meristem exclusion requires the PTGS pathway (Baulcombe and Martin-Hernandez, 2008). It is possible that for a DNA virus, the methylation pathway might be involved in this type of defense.

It is advantageous for plants to keep viral genomes out of the meristem so that there can be no potential infection of its progeny by virus, which might otherwise invade the seed stock. It must also be noted that most viruses are not seed transmitted because hosts typically have strong defense pathways involved in preventing any form of meristem invasion. Meristem cultures are even used to produce virus free plants (Manganaris, 2003). Typically, vegetative meristems prevent the virus from entering into its undifferentiated tissue and infection only spreads to developmentally older areas. The removal of these infected areas causes new growth to occur out of the vegetative

meristem resulting in non-symptomatic tissue, or recovery. While this tissue is by no means virus free, the viral levels are low enough as to reduce symptoms. The same applies to floral meristems. Methylation mutants infected with geminiviruses exhibit stunting as a symptom. Stunting is a result of the virus getting close to the meristem so it can be thought that the virus in these plants might also be getting into the floral meristems and thus into the seeds. It has been discovered that the *Tobbaco rattle virus* encodes a silencing suppressor, 16K, which suppresses plant silencing enough to allow invasion of the meristem in *N. benthamiana* (Baulcombe and Martin-Hernandez, 2008). This study will assess the ability of BCTV and CaLCuV to invade the meristem of *Arabidopsis* by checking for viral DNA in the progeny of infected plants. If the methylation pathway is involved, it is expected that wild-type plants will be able to suppress any attempts at seed transmission/meristem invasion, but methylation mutants should allow some of the virus to make its way into the seed stock of infected plants. Several mutants will be analyzed in this context, including mutants deficient in *ago4*, polymerase IV (*polIV*), or double-stranded RNA binding protein 3 (*drb3*).

Results

Recovery of *Arabidopsis* from infection of mutant L2 BCTV viruses requires *ago4* and *ddc* and is associated with increased cytosine methylation of the viral genome

One of the questions asked was how the removal of infected (primary) tissue from *Arabidopsis* plants affected the growth of new (secondary) tissue. Specifically we wanted to analyze the effect of methylation on the plant's ability to recover successfully.

BCTV was used along with two L2 (L2-1 and L2-2) mutant BCTV viruses. Again, L2 is the silencing suppressor employed by BCTV to knock down plant defenses so it was expected that the mutant L2 viruses would fail to suppress silencing into the secondary growth. Several methylation mutants of *Arabidopsis* were used in this experiment, including *ago4*, *cmt3*, *ddc*, and *drm1/2*. It is important to note that *ddc* is triple mutant that combines the *drm1/2* and *cmt3* mutations. Wild-type plants along with the mutants were inoculated with BCTV and the BCTV mutants, and as expected each virus type induced systemic infections in primary tissue with enhanced symptoms in the methylation deficient plant mutants.

After the removal of the primary infected tissue from wild-type BCTV infected plants, the secondary tissue growth showed continued to display symptoms in all mutants and wild-type plants (Fig. 4A). However, with both of the BCTV L2 mutants, wild-type plants were able to fully recover (Fig. 4B and 4C). Both the *cmt3* and *drm1/2* (not shown) both showed only slight symptoms in their secondary tissue, suggesting intermediate recovery which is most likely due to the redundancy of methyltransferases (Fig. 4B and 4C). However, *ago4* and *ddc* (data not shown) mutant plants failed to recover from infection with either of the L2 mutant viruses. Their secondary tissue still showed severe symptoms suggesting the involvement of *ago4* and *ddc* in recovery (Fig. 4B and 4C).

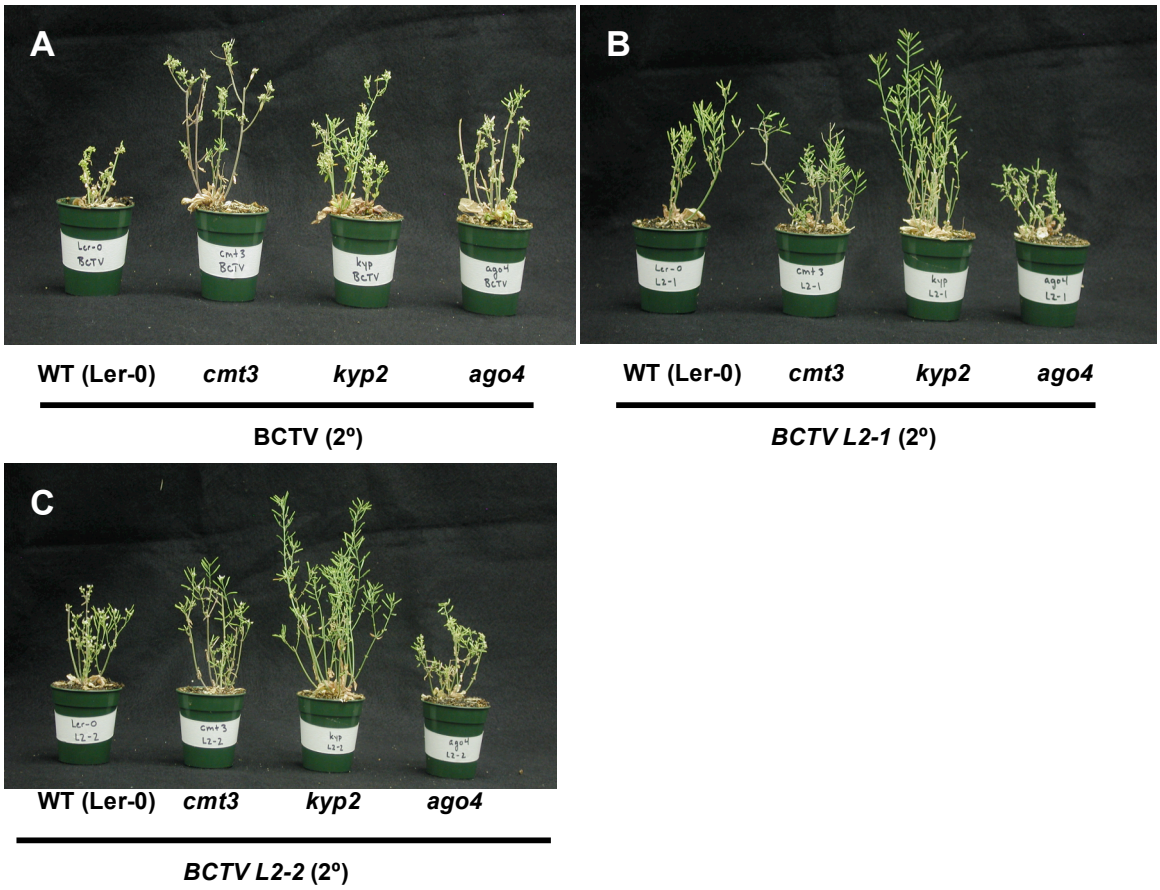

Fig. 4. Recovery of Arabidopsis methylation-deficient mutants from infection with BCTV L2 mutant virus. **A**. Neither WT nor the methylation mutants recover from WT BCTV infection in secondary tissue. **B**. WT, *kyp2*, and to a lesser extend *cmt3* plants recover from *BCTV L2-1* infection while *ago4* does not and still maintains symptomatic tissue in secondary tissue. **C**. Similar to B, but with *BCTV L2-2* mutant virus.

Southern blot analysis confirmed that symptomatic tissue from *ago4* and *ddc* mutant plants contained much higher levels of the mutant virus DNA in both primary and secondary tissue as compared to asymptomatic tissue from similarly infected wild-type plants (Fig. 5). These results suggest that AGO4 and DRM1/2 and CMT3 are integral to recovery, linking recovery with the methylation pathway and the L2 protein with suppression of methylation.

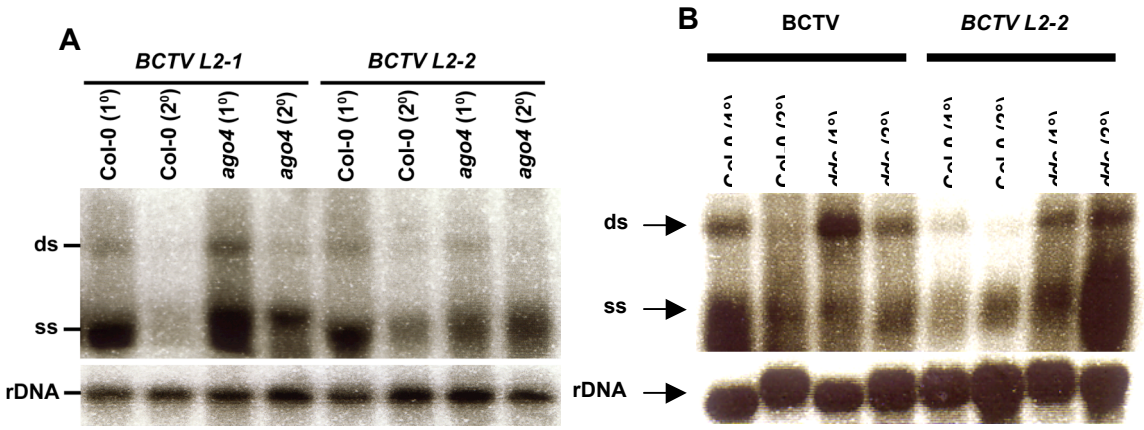

Fig. 5. BCTV DNA levels in recovered and non-recovered tissue. Southern blots are shown. Total DNA extracts obtained from plants infected with BCTV L2 mutants was cleaved with Sca1 to linearize BCTV DNA, and probed with ^{32}P labeled gel purified BCTV DNA. **A**. Plants deficient for *ago4* have higher viral DNA levels in non-recovered secondary tissue than recovered secondary tissue from WT (Col-0) plants. **B**. Plants deficient for *ddc* also have higher DNA levels in non-recovered secondary tissue than recovered secindary tissue from WT plants.

Priya Raja analyzed the methylation of cytosines of the BCTV intergenic region (IR) in secondary infected tissue by using bisulfite sequencing. As would be expected from the visual and southern blot results, mutant L2 BCTV from the secondary tissue of wild-type plants was heavily methylated resulting in suppression of the virus and correlated with recovery. Secondary tissue from *ago4* showed drastically decreased cytosine methylation with mutant L2 BCTV, similar to that of wild-type BCTV infected tissue (Fig. 6).

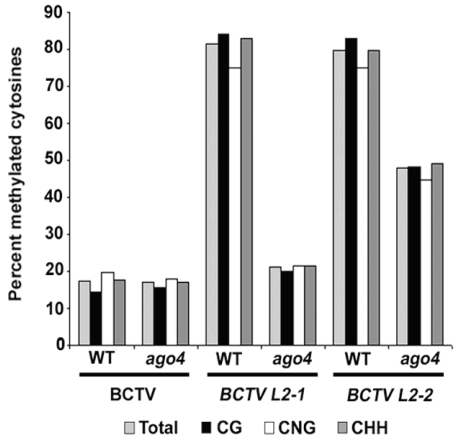

Fig. 6. Viral DNA in recovered tissue is highly methylated. The histogram summarizes cytosine methylation levels in different sequence contexts, as determined by bisulfite sequencing of the BCTV IR region. Note hypermethylation of BCTV L2 mutant DNA in recovered tissue from WT plants.

The results of the recovery experiments described here are summarized in Table 1. These data clearly indicate that the AGO4-mediated methylation pathway, and cytosine methylation by the functionally redundant DRM1/2 and CMT3 methyltransferases, are required for recovery of Arabidopsis plants from geminivirus infection.

Table 1. Summary of recovery experiments.

Methylation Mutant	Function	Recovery after mutant L2 BCTV infection (removal of primary tissue)
Col-0	WT	Yes
Ler-0	WT	Yes
ago4	Component of the RNA-induced silencing complex (RISC)	No, severe symptoms
ddc	Triple *drm1/2* and *cmt3* mutant, no *de novo* cytosine methylation except CG	No, severe symptoms
cmt3	cytosine methyltransferase, primarily involved in CNG methylation	No, only slight symptoms
kyp2	H3K9 methyltransferase	Yes

PolIV and DRB3 required for virus exclusion from *Arabidopsis* seeds and floral meristem exclusion

A second question asked was whether methylation is involved in preventing geminiviruses from entering into the plant's seed stock. In order for the virus to pass into the seed, it must make its way into the floral meristem from which it is normally excluded. Thus viral seed invasion is dependent upon viral invasion of the floral meristem. Wild-type, *polIV*, and *drb3 Arabidopsis* plants were inoculated with CaLCuV and after the onset of infection, the plants were taken to seed and the seeds were collected and pooled together. From this pool, batches of one flat per mutant (~32 plants) were planted,,allowed to grow normally (1^{st} generation plants), and observed for symptomatic tissue. The first batch of *polIV* plants from inoculated parental seeds showed 13/25 plants with symptomatic tissue and that tissue could only be considered slightly symptomatic with slight crinkling of leaves on the stalks or rosettes. The first batch of *drb3* plants showed 8/20 plants with symptomatic tissue similar to *polIV*.

The ten most symptomatic plants had their floral tissue harvested individually and the DNA amplified via polymerase chain reaction (PCR) with CaLCuV specific primers (386 bp fragment amplified). These samples were then analyzed by southern blot using a 32P-labeled probe to detect CaLCuV DNA. Of these, 8/10 *drb3* and 7/10 *polIV* samples showed evidence of DNA (Fig. 7A and 7B). The PCR amplification was necessary

because of the extremely small amount of viral DNA that made it into the seeds. Southern blots run without amplification showed no hybridization. Curiously, additional larger and smaller bands also appeared. These are likely amplification products from concatameric (multimeric) and subgenomic viral DNAs, respectively.

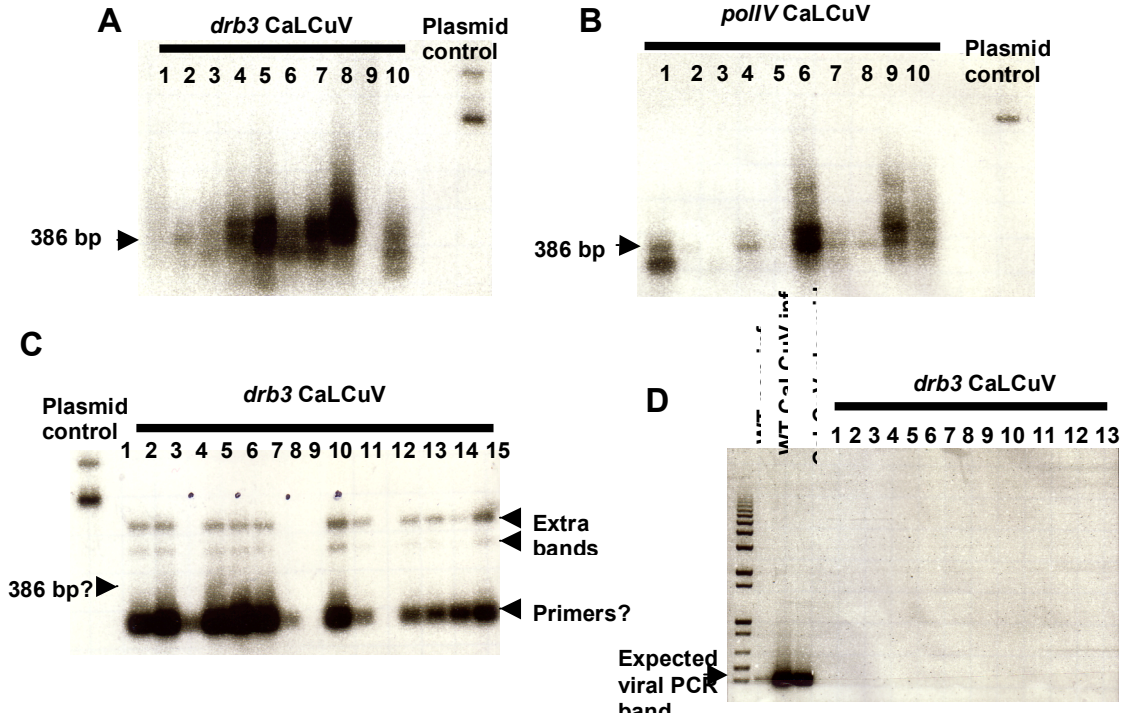

Fig. 7. Viral DNA is seed-transmitted in mutant but not wild-type plants. PCR products were subjected to Southern blots analysis using a gel purified, ^{32}P-labeled, CaLCuV A fragment as probe. **A**. 1st generation *drb3* plants contain viral DNA; 8/10 plants show the 386 bp virus fragment. **B**. 1st generation *polIV* plants contain viral DNA; 7/10 plants show the 386 bp virus fragment. **C**. An additional group of non-symptomatic *drb3* 1st generation plants showing higher molecular weight viral DNA bands and little or no 386 bp product. **D**. An unsuccessful repeat of the experiment shown in C, with some additional samples. The band apparent in the non-infected WT sample is probably due to spill over from the neighboring lane.

A second batch of *drb3* mutants tested in a similar fashion were nearly devoid of symptomatic tissue, but southern blot analysis with PCR amplification found 11/15 plants showing hybridization. Interestingly, though the expected 386 base pair band was absent and several extra bands were in its place (Fig. 7C). These larger bands may be derived from concatameric (multimeric) viral genomes. Further work is needed to confirm this.

To confirm the presence of viral DNA in first generation plants, we looked to a method employed by the TempliPhi (GE Healthcare) kit which utilizes the bacteriophage Φ29 DNA polymerase and amplifies through rolling circle replication (Inoue-Nagata, et al., 2004) (Fig. 8). This ensured that the only DNA being amplified would be the circular viral DNA.

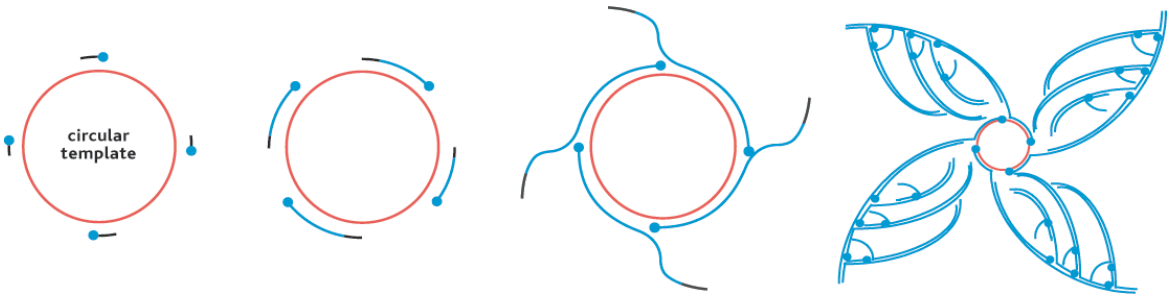

Fig. 8. Graphic of the mechanism of TempliPhi amplification. Random hexamer primers sit on the circular template and the bacteriophage φ29 DNA polymerase produces copies which are in turn primed and copied. This polymerase is highly capable of strand displacement. This is repeated on each new copy until the nucleotide pool is exhausted.

A third batch of *polIV* (10) and *drb3* (14) first generation samples were subjected to TempliPhi amplification along with 26 Col-0 individual plant samples. This batch of plants again showed very little in the way of symptomatic tissue. Initial Southern blots of TempliPhi products showed that three *polIV* and four *drb3* samples had hybridization (Fig. 9A). Two bands appeared, both of quite a large size and we were unsure of what

they may have been, so the TempliPhi reaction was repeated and the products cut with EcoR1 to linearize and excise unit-length copies of CaLCuV DNA. A subsequent blot of the hybridization positive samples showed what appeared to be unit-length products as well as a larger band that likely consists of two or more unit-length genomes. (Fig. 9B). Importantly, no evidence of viral DNA was seen in samples from 13 first generation, WT (Col-0) plants (Fig. 9C). The absence of viral DNA from wild-type plants indicates that methylation plays an important role in preventing the virus from entering the meristem .

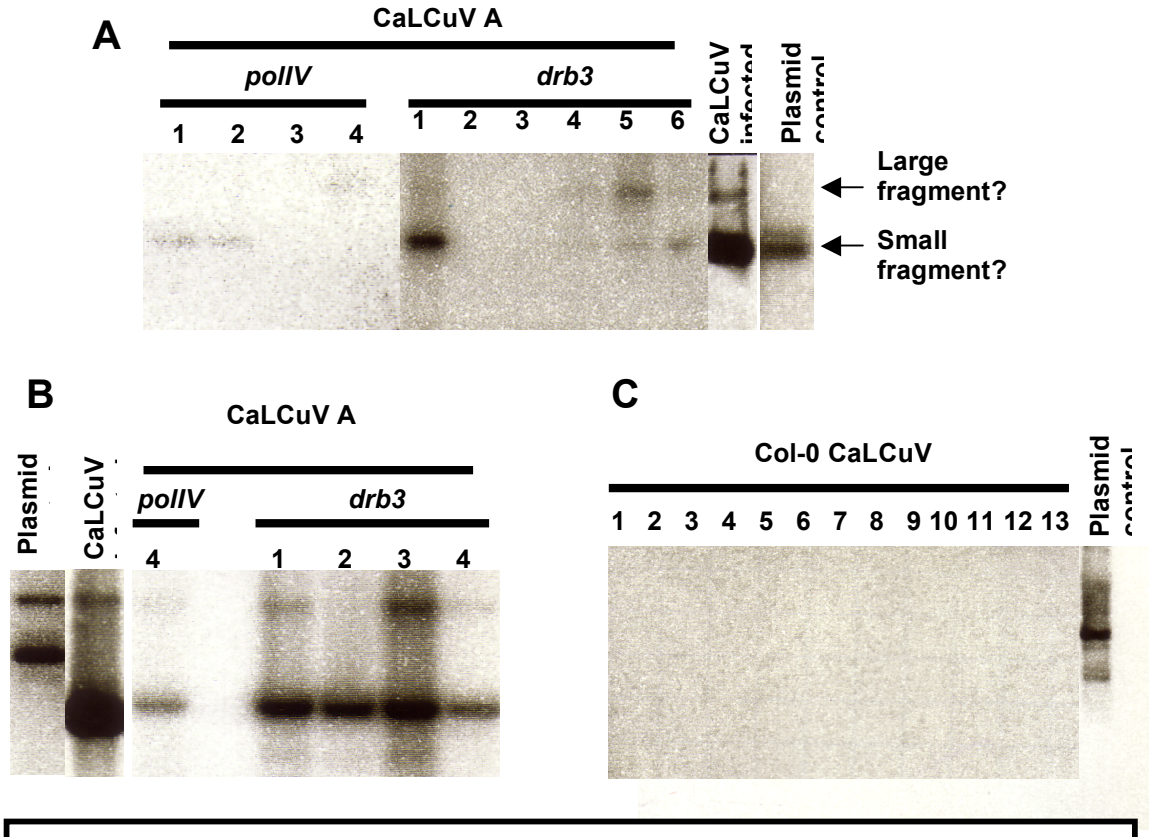

Fig. 9. Circular viral DNA is present in mutant, but not wild type, first generation plants. TempliPhi products were subjected to Southern blot analysis using a CaLCuV probe. A. Uncut TempliPhi product from 1st generation *polIV* and *drb3* mutant plants. **B**. TempliPhi products from *polIV* and *drb3* mutant plants cut with EcoR1. A unit-length viral band is seen and a larger band likely to be concatamers of TempliPhi product. **C.** No TempliPhi products were detected from first generation WT (Col-0) plants, indicating that CaLCuV does not travel through WT seeds.

These results have established that geminiviruses can be transmitted through the seeds of *Arabidopsis* at some frequency. Taking any positive TempliPhi hits as the most accurate method of confirming viral transmission, CaLCuV transmits at a rate of 30% in *polIV* and at 29% in *drb3* (Table 2). Of course additional screenings will need to be conducted to get a more accurate number, but it is nonetheless significant. The presence of viral transmission in methylation mutants, but not WT plants, suggests that methylation defense is important in preventing geminiviruses from invading the meristem of *Arabidopsis*. Furthermore, the presence of viral transmission in *drb3* adds to the case that this protein is involved in the methylation pathway, but additional studies are needed to confirm this.

Table 2. Transmission of CaLCuV DNA through seed.

Methylation Mutant/Wild-Type	Plants grown with seeds from infected plants, symptomic tissue?	Frequency of transmission (measured by southern blot, TempliPhi and PCR)	Frequency of transmission (TempliPhi only) (more accurate?)
Col-O (CaLCuV)	No	0% (26 plants)	0% (26 plants)
Ler-O (BCTV)	No	?	?
drb3 (CaLCuV)	Some, mild (8/20 plants)	50% (12/24 plants)	30% (3/10 plants)
polIV (CaLCuV)	Some, mild (13/25 plants)	50% (10/20 plants)	29% (4/14 plants)
ago4 (BCTV)	No	?	?
kyp2 (BCTV)	No	?	?
cmt3 (BCTV)	No	?	?
ddc (BCTV)	?	?	?

The next question to be asked is why the virus that is present in the seeds is not being amplified to bring about a full fledged infection. Work is planned in the form of

challenging these 1st generation plants to a subsequent inoculation with fresh virus. Addition of fresh virus might be easily suppressed by plants in which seed transmission has occurred because they have already "seen" the virus and may be better equipped to guard against infection. We expect that WT plants, which we have shown as not being able to transmit geminiviruses through the seed, to be completely naïve to the introduction of new virus which should result in a typical infection. However, perhaps methylation mutants that have failed to keep the virus out of the meristem will have some ability to suppress a new infection. Ultimately we are questioning whether the presence of virus in seeds gives some sort of immunity to future infections.

Conclusion and Future Work

The above results indicate the importance of the *Arabidopsis* methylation pathway in the defense against geminiviruses. While the two sections may seem rather unrelated, in actuality, both are involved in a common theme; the prevention of geminiviruses from entering the meristem. The recovery experiments show that the lack of significant methylation proteins allows for the entry of the virus into the meristem so that recovery does not take place upon the removal of symptomatic tissue. Recovery is also helpful in showing the link between L2 with the methyl cycle and thus helping to solidify methylation as an integral plant defense. The seed transmission experiments show that the lack of particular methylation proteins allows for the virus to enter into a plant's seed stock, something that could not be considered advantageous. Obviously additional work needs to be done, especially in the seed transmission project. Large samples of plants need to be subjected to TempliPhi amplification to achieve a more accurate transmission

rate. Additional transmission tests have currently been initiated using BCTV with *ago4*, *cmt3*, *kyp2*, and *ddc* mutants. Several 1st generation seeds were also sent to Dr. Jane Polston (University of Florida) to see if the virus present in first generation plants can be transmitted by whiteflies to naïve plants to cause infection. This will help us to determine if virus particles are present in the first generation plants, and to understand why there is a suppression of virus replication after it has been seed transmitted.

Materials and Methods

***Arabidopsis* Mutants**

Wild-type Ler-0 (CS20) Landsberg 1 erecta ecotype; *ago 4-1 gl1-1* (CS6364/ At2g27040) argonaute 4; *kyp2 gl1-1* (CS6367/ At5g13960) kryptonite/SuVH4; *cmt3-7* (CS6365/ At1g69770) chromomethylase; Wild-type Ws-2 (CS22659) Wassilewskija ecotype; *drm1/drm2* (CS6366/ At1g28330 and At5g14620) domains rearranged methyltransferase; Wild-type Col-0 (CS60000) Columbia ecotype; *polIV (nrpd2a-1)* (SALK_095689/ At3g23780) nuclear RNA polymerase D 2A; *drb3 (rbp3)* (SALK_022644/ At3g26932) double-stranded RNA binding protein.

***Arabidopsis* Virus Inoculation**

Agrobacterium tumefaciens cultures of tandem repeated CaLCuV and BCTV (WT, L2-1, and L2-2) were grown overnight. Plants were inoculated within five days of bolting. The bolts were cut at their emergence from the rosette and the inoculum was added to the newly exposed tissue and was then stabbed with an insect pin 15-20 times. CaLCuV symptoms were typically seen 14-21 days post-inoculation and the tissue was also

harvested at this time. BCTV symptoms were seen slightly later at 21-30 days post-inoculation and the infected tissue was also removed at the same time. Symptoms were seen in the floral tissue of the plant. For the recovery experiment, samples were pooled together from 4-5 infected plants. For the seed transmission experiment, samples were restricted to individual plants as to ascertain a frequency of transmission and to avoid cross contamination. Also for the recovery experiment, primary infected tissue was removed and allowed to continue growing under normal conditions after which the new secondary tissue was removed 14-21 days later.

Arabidopsis DNA Extraction

Infected floral tissue was removed in approximately 250 mg samples and frozen in liquid nitrogen and stored at -80°C. Extraction began with the grinding of the infected tissue with mortar and pestle in liquid nitrogen and use of DNeasy columns (Qiagen) to extract DNA.

PCR

PCR done with CaLCuV A primers and the samples were either uncut or cut with EcoR1 depending upon the experiment. The PCR was done with *taq* polymerase at the following conditions; a three part cycle consisting of a 45 second 95°C denaturing phase, 45 second 55°C annealing phase, and a one minute 72°C extension phase, with the cycle being repeated 25 times. The primers used were CaLCuV2556UF, GGGGATATGTTAAGAATATATTTCGGG and CaLCuV359UR, TCCCCGCCATAGAACGCCAC;

TempliPhi

Based on Inoue-Nagata et. al., 2004. TempliPhi consisted of the use of the Amersham Biosciences TempliPhi kit which involved the addition of a sample buffer to the extracted DNA in 10-20 ng amounts and denatured at 95°C. Then a reaction buffer and bacteriophage φ29 DNA polymerase were added and the reaction was allowed to proceed for at least 16 hours at 29°C. The goal of the kit is to amplify circular DNA only (ds or ss) through random hexamer priming and rolling circle amplification.

Southern Blots

The samples were run on a 1% electrophoresis gel and then washed with 1 M HCl, a 1.5 M NaCl/0.5 M NaOH denaturant, and a 1.5 M HCl/0.5 M Tris (pH 7.5) neutralizer each for thirty minutes. An overnight transfer of the DNA onto a Nytran Super Charge membrane with 10 X SSC followed the washes. The blot was then UV cross linked (1700 μJ) and hybridized overnight at 42°C with a ^{32}P full length virus probe in Ultrahyb hybridization buffer (Ambion). The probe was synthesized using a Strip-EZ DNA kit (Ambion). A Phosphorimager (Bio-Rad Molecular Imager FX) measured the signal intensity.

Bisulfite sequencing.

The method is based on Frommer *et al*. (16). DNA isolated from infected plant tissue was linearized overnight using appropriate restriction enzymes. Proteinase K digestion was subsequently carried out overnight followed by bisulfite conversion using C-T

conversion reagent (EZ-DNA Methylation Gold; Zymo research). Primers were designed against converted template and the intergenic region of the viral genome was amplified by PCR. PCR product was purified using Promega Wizard columns, TA cloned, and individual clones were sequenced at the Ohio State University Plant Microbe Genomics Facility. For conversion control, plasmids containing BCTV DNA were added to a vast excess of healthy plant DNA extract and treated with bisulfite reagent. The following forward (F) and reverse (R) primers were used to amplify BCTV intergenic regions (IR) following bisulfite conversion: BCTV2640CF, GGGATATGTAAGAAATATG and BCTV147CR, TCTCCCCTTCTATTAACCAATCAAC.

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